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NOTES

Possible Role for the Essential GTP-Binding Protein Obg in Regulating the Initiation of Sporulation in *Bacillus subtilis*

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We fused *obg*, encoding an essential GTP-binding protein in *Bacillus subtilis*, to the LacI-repressible, IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter Pspac. Depletion of Obg, following removal of IPTG, caused a defect in sporulation and in expression of sporulation genes that are activated by Spo0A~P. These defects were significantly relieved by a mutation in *spo0A* (*rvtA11*) that bypasses the normal phosphorylation pathway, indicating that Obg might normally be required, either directly or indirectly, to stimulate activity of the phosphorelay that activates Spo0A.

The initiation of sporulation in *Bacillus subtilis* is controlled, in part, by a multicomponent phosphorelay that results in the phosphorylation, and hence activation, of the transcription factor encoded by *spo0A* (4). Several histidine protein kinases are involved in the initiation of sporulation (1, 20, 22, 29). Phosphate is transferred from the kinases to Spo0F, from Spo0F to Spo0B, and finally from Spo0B to Spo0A (4). A threshold mechanism controls the initiation of sporulation (5), and the phosphorelay functions to integrate many of the signals that control initiation, including nutrient deprivation, DNA replication, DNA damage, chromosome partitioning, and Krebs cycle signals (13–18). In addition to the phosphorelay, several other proteins are known to regulate production and accumulation of Spo0A~P. These proteins include the phosphatase encoded by *spo0E* (28, 31), the transcriptional regulator encoded by *sinR* (2, 25, 26), the oligopeptide permease encoded by *spo0K* (30, 33), and the proteins encoded by *soj* and *spo0J* (16).

One protein that has been postulated to control the phosphorelay is the G protein encoded by *obg* (42). The *obg* gene product has been purified and shown to bind GTP (40) and to have GTPase activity (42). *obg* is immediately downstream from and cotranscribed with *spo0B* (6), is essential for vegetative growth, and is required for efficient sporulation (21). Because of its association with *spo0B*, it has been tempting to imagine that Obg has a role in regulating the activity of the phosphorelay and perhaps of Spo0B (4, 8, 12).

A conditional allele of *obg*. To test the effects of *obg* on growth and sporulation, we made a conditional allele of *obg* such that the only copy of the gene was under the control of the LacI-repressible, IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter Pspac (11, 45). We cloned *obg* after PCR amplification using primers that were designed on the basis of the published DNA sequence (3, 6). A 197-bp fragment of *obg*, extending from 70 bp upstream of the putative start codon to the *Aat*II site at codon 43 (3, 6), was sequenced to be sure that there were no mutations introduced during the PCR amplification and cloning. This fragment was then cloned downstream of Pspac in the integrational vector pDH88 (11) to give pVS7 (Fig. 1). pVS7 was integrated into the chromosome of *B. subtilis* by selecting for chloramphenicol resistance in the presence of IPTG, allowing expression of the intact *obg* gene from Pspac (Fig. 1). The Pspac-*obg* strain (SJV6) required IPTG for growth; concentrations of IPTG of 100 μM or higher resulted in growth and viability that were indistinguishable from those of the wild type (data not shown).

***obg* is required for expression of Spo0A-controlled genes.** We used the Pspac-*obg* fusion to test for a possible role of *obg* in sporulation. Cells were grown for several generations in the presence of IPTG and then resuspended in fresh medium without IPTG. Without IPTG-induced transcription, the levels of Obg in the cell would be depleted during subsequent growth. Cells appeared to grow normally in medium without IPTG for approximately three generations before there was any noticeable change in the growth curve compared with that for the same strain grown in the presence of 100 μM IPTG (Fig. 2). While the growth rate decreased and eventually leveled off, there was no detectable effect on viability for at least 6 h after the removal of IPTG. In an experiment similar to the one for which the results are shown in Fig. 2, the number of CFU per optical density unit at 600 nm (OD_{600}) 1 h after the removal of IPTG was 8.5×10^7, while the number of CFU per OD_{600} 6 h after the removal of IPTG was 7.1×10^7. During exponential growth in the presence of IPTG, the number of CFU per OD_{600} was approximately 8×10^7.

Depletion of Obg from cells caused a defect in the expression of early sporulation genes. We monitored transcription of a *spoIIE-lacZ* fusion in cells containing the Pspac-*obg* fusion in the presence and absence of IPTG. Transcription of *spoIIE* normally begins shortly after the initiation of sporulation and is directly activated by Spo0A~P (10, 46). Cells were first grown for several generations in the presence of IPTG, removed from the growth medium by centrifugation, resuspended in fresh medium, and divided into two parts, one with no IPTG and the other with 100 μM IPTG. Sporulation was induced by the addition of mycophenolic acid (32 μg/ml) at various times after the removal of IPTG. Mycophenolic acid inhibits IMP dehydrogenase, causing a decrease in GMP, GDP, and GTP and resulting in the induction of sporulation (7). Preliminary ex-

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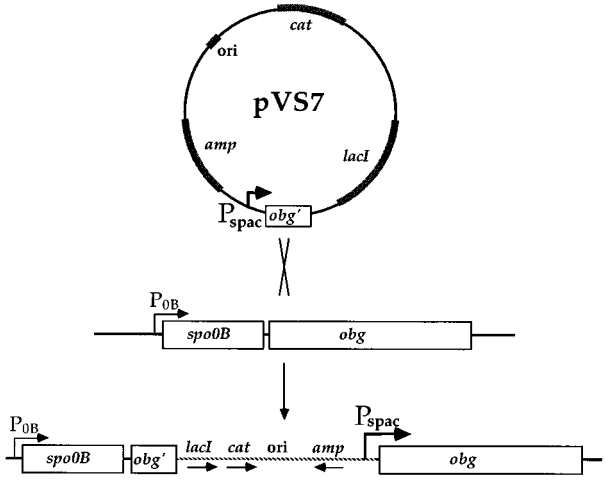

FIG. 1. pVS7 and Pspac-*obg*. pVS7 was made by cloning a fragment of *obg* into pDH88 (11). The *obg* fragment is 197 bp and extends from 70 bp upstream of the putative start codon (3, 6) to the *Aat*II site at codon 43. pNG10 contains this fragment in pBluescriptII SK (Stratagene) between *Xba*I and *Bam*HI. The *Xba*I-to-*Cla*I fragment from pNG10 that contains the *obg* fragment was subcloned into pDH88 between *Xba*I and *Cla*I to give pVS7. pNG10 was used to sequence the fragment of *obg*. pVS7 was recombined into the *obg* locus (*obg*::pVS7) by single crossover selecting for Cm^r and generated Pspac fused to an intact *obg*. The figure is not drawn to scale.

periments indicated that 2.5 to 3.5 h after the removal of IPTG was the optimal time to observe the effects on sporulation before growth was significantly impaired.

Expression of *spoIIE-lacZ* was greatly decreased when sporulation was induced 3 h after the removal of IPTG (Fig. 3A). Three hours after the initiation of sporulation, accumulation of β-galactosidase-specific activity in the culture with no IPTG was only 10 to 20% of that in the culture with 100 μM IPTG (Fig. 3A). A similar effect on the transcription of *spoIIA* was observed (Fig. 3B). These results indicate that depletion of Obg from cells causes a defect in expression of early sporulation genes and that Obg might normally be required for efficient initiation of sporulation and production of Spo0A~P.

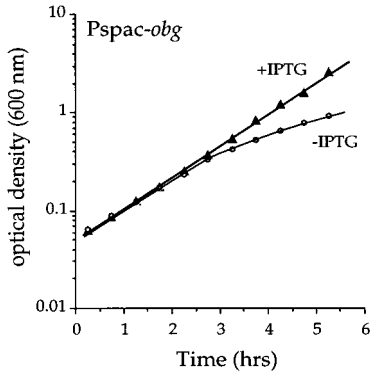

FIG. 2. Growth of Pspac-*obg* strain in the presence (+) and after removal (−) of IPTG. Strain SJV27 [JH642 *obg*::pVS7 (Pspac-*obg*) *spoIIE-lacZ spo0A*$^+$-*spc*] was grown overnight at room temperature as a lawn on LB plates with IPTG (100 μM) and chloramphenicol (5 μg/ml). The cells were then used to inoculate a culture in $S7_{50}$ minimal medium (19, 41) containing glucose (1%), glutamate (0.1%), tryptophan (40 μg/ml), phenylalanine (40 μg/ml), and IPTG (100 μM). The culture was started at OD_{600} of ~0.05 and grown to OD_{600} of ~0.7. The cells were removed by centrifugation, resuspended, and diluted to OD_{600} of ~0.05 in the presence (100 μM) or absence of IPTG. The OD_{600} was monitored as a function of time after resuspension.

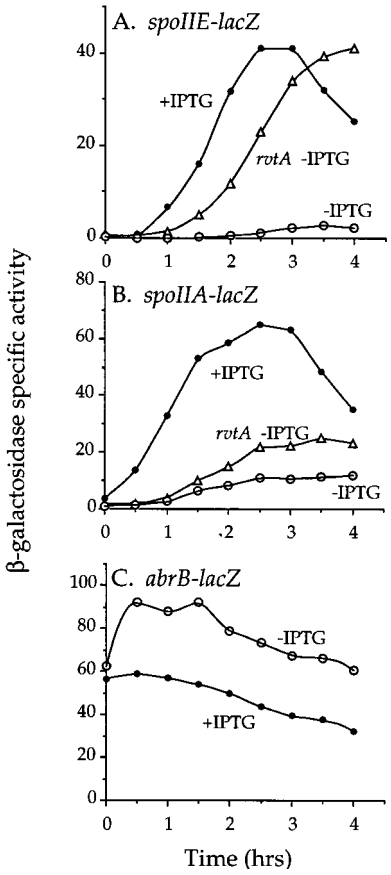

FIG. 3. Expression of *spoIIE* (A), *spoIIA* (B), and *abrB* (C) in Pspac-*obg* strains. The indicated strains were first grown in the presence of IPTG (100 μM) as described in the legend to Fig. 2. Cells were removed from culture by centrifugation and resuspended at an OD_{600} such that after 3 to 4 h of growth they would be at a density of ~0.5 to 0.9. Mycophenolic acid (32 μg/ml) was added (time zero) to induce sporulation (7) 3 to 4 h after resuspension. β-Galactosidase-specific activity was determined by standard methods (19, 27) and is expressed as (ΔA_{420} per minute per milliliter of culture per OD_{600}) × 1,000 (27). *spoIIE-lacZ* (*spoIIE*$^+$::pZΔ326-GV39 *cat*::*neo*) (10) and *spoIIA-lacZ* (*spoIIA*$^+$::pPP81 *cat*::*neo*) (43) are transcriptional fusions made by integrating the indicated plasmids (containing the *lacZ* fusions) by a single crossover into the *spoIIA* and the *spoIIE* loci, respectively. The constructs have been modified to confer resistance to neomycin (14). The *abrB-lacZ* fusion integrated at the *amyE* locus was a gift from M. Strauch (38), and the *cat* marker had been converted to *neo* (23). The *rvtA11* mutation was introduced into strains with a linked *spc* marker (9, 14). The presence of *rvtA11* was confirmed by transforming DNA from the appropriate strains into an *spo0F* mutant, selecting for Spc^r, and testing for cotransformation of the *rvtA11* mutation as judged by a Spo$^+$ phenotype. *rvtA11* and *spc* are ~80 to 90% linked by transformation. (A) Expression of *spoIIE-lacZ* in strain SJV27 [JH642 *obg*::pVS7 (Pspac-*obg*) *spoIIE-lacZ spo0A*$^+$-*spc*] with IPTG (filled circles) and without IPTG (open circles) and strain SJV26 [JH642 *obg*::pVS7 (Pspac-*obg*) *spoIIE-lacZ rvtA11-spc*] without IPTG (open triangles). Mycophenolic acid was added 3.25 h after resuspension of the cells in the presence or absence of IPTG. Similar results were obtained in several independent experiments with sporulation induced from 3 to 3.25 h after resuspension of the cells. (B) Expression of *spoIIA-lacZ* in strain SJV25 [JH642 *obg*::pVS7 (Pspac-*obg*) *spoIIA-lacZ spo0A*$^+$-*spc*] with IPTG (filled circles) and without IPTG (open circles) and strain SJV24 [JH642 *obg*::pVS7 (Pspac-*obg*) *spoIIA-lacZ rvtA11-spc*] without IPTG (open triangles). Mycophenolic acid was added 3 h after resuspension of the cells in the absence or presence of IPTG. Similar results were obtained in three independent experiments. (C) Expression of *abrB-lacZ* in strain SJV55 [JH642 *obg*::pVS7 (Pspac-*obg*) *amyE*::(*abrB-lacZ cat*::*neo*) *spo0A*$^+$-*spc*] with IPTG (filled circles) and without IPTG (open circles). Mycophenolic acid was added 4 h after resuspension of the cells in the absence and presence of IPTG. Similar results were obtained when mycophenolic acid was added 3 h after resuspension. The level of expression of *abrB-lacZ* in the *rvtA* mutant was very low (data not shown).

TABLE 1. Sporulation of Pspac-*obg* strains in the presence and absence of IPTG[a]

Strain (relevant genotype)[b]	No. of viable cells/ml		No. of heat-resistant spores/ml (% sporulation)	
	−IPTG	+IPTG	−IPTG	+IPTG
SJV27 (*spo0A*$^+$)	3.7×10^8	7.5×10^8	7.0×10^5 (0.19)	1.5×10^8 (20)
SJV26 (*rvtA11*)	6.4×10^8	8.7×10^8	2.2×10^7 (3.4)	1.7×10^8 (20)
SJV34 (*spo0A*$^+$)	2.8×10^8	5.0×10^8	4.0×10^5 (0.14)	2.0×10^8 (40)
SJV33 (*rvtA11*)	1.8×10^9	6.3×10^8	4.3×10^7 (2.4)	2.0×10^8 (32)

[a] Cells were grown in defined minimal medium as described in Fig. 3. Sporulation was induced by the addition of mycophenolic acid (32 μg/ml) 3 h after resuspension of the cells in either the presence (+) or absence (−) of IPTG. The number of total viable cells (CFU per milliliter) and heat-resistant spores (80°C for 15 min) (CFU per milliliter) was determined 21 h after the addition of mycophenolic acid. Samples were put on LB plates containing chloramphenicol (5 μg/ml) to ensure presence of the Pspac-*obg* fusion and IPTG (1 mM) to induce expression of Pspac-*obg*. Similar results were obtained in several independent experiments.

[b] All four strains were derived from JH642 (*trp phe*) and contain the Pspac-*obg* fusion (*obg*::pVS7) and an *spc* marker that had been placed downstream of the indicated *spo0A* allele (9, 14). SJV26 and SJV27 are described in Fig. 3. SJV33 and SJV34 contain an *ald-lacZ* fusion (36).

The decreased expression of *spoIIE* and *spoIIA* was not simply an effect of decreased viability or an overall decrease in transcription due to depletion of Obg. Accumulation of β-galactosidase-specific activity from an *abrB-lacZ* fusion was not reduced in the Pspac-*obg* strain in the absence of IPTG. Rather, it was slightly higher than that in the same strain in the presence of IPTG, even when sporulation was induced 4 h after the removal of IPTG (Fig. 3C). Transcription of *abrB* is directly repressed by Spo0A∼P (32, 39), and the continued or slightly increased expression of *abrB* after depletion of Obg is consistent with a role for Obg in activating the phosphorelay.

Depletion of Obg also caused a defect in the production of heat-resistant spores while having little or no effect on cell viability. The sporulation frequency in the culture with no IPTG was reduced to approximately 1% of that in the culture with 100 μM IPTG (Table 1). Under these conditions, there was little or no effect on cell viability for at least 21 h after the initiation of sporulation. Cell viability and sporulation were determined by the number of CFU on LB plates containing IPTG (1 mM) and chloramphenicol (5 μg/ml). The decrease in sporulation frequency caused by depletion of Obg was consistent with the recent finding that a temperature-sensitive mutation in *obg* causes a defect in sporulation (21).

Obg effects on sporulation are partly bypassed by the *rvtA11* mutation in *spo0A*. Because depletion of Obg inhibited expression of genes activated by Spo0A∼P (Fig. 3), it seemed possible that Obg might be required for activation of the phosphorelay. Mutations in *spo0A* that bypass the phosphorelay (e.g., *sof* and *rvtA*) (24, 34, 35, 37) were found to partly bypass the effect of depleting Obg. The *rvtA11* missense mutation in *spo0A* bypasses the need for *spo0F* and *spo0B* and appears to allow Spo0A to obtain phosphate directly from KinC (20, 22). Growth of the Pspac-*obg* strain in the absence of IPTG was not detectably affected by the *rvtA11* mutation, compared with *spo0A*$^+$, indicating that *rvtA11* did not suppress the growth phenotype caused by depletion of Obg. However, the *rvtA11* mutation significantly restored expression of *spoIIE* and *spoIIA* in the Pspac-*obg* strain in the absence of IPTG (Fig. 3). Accumulation of β-galactosidase-specific activity from the *spoIIE-lacZ* fusion in the *rvtA11* strain in the absence of IPTG was typically 60 to 100% of that in the *spo0A*$^+$ strain in the presence of IPTG. The slight delay in gene expression under these conditions in the *rvtA11* mutant is similar to the delay in gene expression caused by *rvtA11* under other conditions that inhibit activity of the phosphorelay (14). The effect of the *rvtA11* mutation on expression of *spoIIA* was not as great as the effect on *spoIIE*. This difference might be due to the fact that *spoIIA* is transcribed by RNA polymerase containing sigma-H (43, 44) and that *spoIIE* is transcribed by RNA polymerase containing sigma-A (46).

In addition to the effect on sporulation gene expression, the *rvtA11* mutation also partly suppressed the sporulation defect caused by depletion of Obg. We determined the number of heat-resistant spores per milliliter after induction of sporulation with mycophenolic acid in the Pspac-*obg* strains with the *rvtA11* mutation (Table 1). In the two experiments, in the absence of IPTG sporulation was at least 10-fold greater in the *rvtA11* mutant than in the otherwise isogenic *spo0A*$^+$ strain (Table 1). Similar results were obtained in several independent experiments. The effects on sporulation were not due to effects on cell viability. Under the conditions of the experiment, cells remained viable for at least 21 h after the initiation of sporulation (Table 1).

Our results indicate that Obg is required to stimulate the activation of Spo0A, perhaps by regulating one of the components of the phosphorelay. Obg could directly interact with a component of the phosphorelay (Spo0B?) to modulate the initiation of sporulation in response to a vital cellular process, perhaps DNA replication (14) or chromosome partitioning and cell cycle (16). Alternatively, the effects of Obg on the phosphorelay could be indirect, via effects on an essential cellular function. The analysis of a temperature-sensitive *obg* mutant indicated that Obg was not required for general transcription or translation (21), assuming that the mutant allele encoded a temperature-sensitive protein and was not temperature sensitive only if synthesized at the nonpermissive temperature. If Obg is required for the initiation of DNA replication as suggested (21), then its effects on the phosphorelay and the initiation of sporulation are consistent with previous results suggesting a link between the phosphorelay and the initiation of DNA replication (14).

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